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Establishing and Characterizing a CD30-Positive Cell Line Harboring HHV-8 From a Primary Effusion Lymphoma

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Primary effusion lymphoma (PEL, or body-cavity-based lymphoma [BCBL]) is a new subtype of non-Hodgkin's lymphoma in which tumor cells locate in the body cavity exclusively. PEL/BCBL is widely accepted as one of the neoplastic complications of AIDS, associated mostly with human herpesvirus 8 (HHV-8/Kaposi's sarcoma-associated herpesvirus [KSHV]) and Epstein-Barr virus (EBV). We established and characterized a PEL cell line named TY-1 from a 47-year-old patient with AIDS. TY-1 exhibits indeterminate immunophenotype, expressing CD45 and CD30 cell surface antigens but not expressing B- or T-cell markers. Cytogenetic analysis revealed the representative karyotype of 50,XYq-,+7,+8,+11,+15. Southern blot analysis demonstrated HHV-8 and EBV genomes in the original tumor cells obtained from the pericardial effusion, while HHV-8 but not EBV was detected in TY-1 using PCR or Southern blot analysis. Tetradecanylphorbol acetate treatment induced some TY-1 cells to proceed to the reproductive phase. This cell line may be an useful tool for research on PEL and HHV-8. ***J. Med. Virol. 58:394–401, 1999.***

KEY WORDS: primary effusion lymphoma (PEL); Kaposi's sarcoma-associated herpesvirus (KSHV); human herpesvirus 8 (HHV-8); Epstein-Barr virus (EBV); CD30

INTRODUCTION

Primary effusion lymphoma (PEL) is a rare neoplastic complication of AIDS, exhibiting unique characters in clinical course, immunophenotype and molecular aspects [Cesarman et al., 1995a; Nador et al., 1995]. PEL has been described as growing in the pleural, pericardial, and peritoneal cavities as lymphomatous effusions, with no identifiable tumor mass throughout the clinical course of the disease [Cesarman et al., 1995a; Nador et al., 1996].

HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a new type of herpesvirus identified from AIDS-associated Kaposi's sarcoma [Chang et al., 1994]. The HHV-8 is often, if not always, demonstrated in PELs associated with AIDS [Cesarman et al., 1995a; Nador et al., 1996]. In these PELs, the Epstein-Barr virus (EBV) is a frequent coinfection.

Several cell lines have been established from PEL. They are mostly infected with both HHV-8 and EBV simultaneously [Cesarman et al., 1995b; Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996]. These viruses are mostly in the latent phase, with very limited numbers of HHV-8-producing cells. They can, however, easily be induced to the lytic phase by treatment with tetradecanoylphorbol acetate (TPA) [Renne et al., 1996; Miller et al., 1997]. Among such recently established PEL cell lines, BCBL-1 and BC-3 have been shown to be infected with HHV-8 but not with EBV [Arvanitakis et al., 1996; Renne et al.,1996]. The BCP-1 cell line, established from peripheral blood of a patient with PEL, has also been reported to be EBV-negative and HHV-8-positive [Boshoff et al., 1998]. All these PEL cell lines, whether EBV-negative or EBV-positive, were reported to be of B-cell lineage, lacking the expression of CD19 and CD20.

We now report the establishing and characterizing of a new HHV-8-positive/EBV-negative PEL cell line, named TY-1. This cell line was established from the pericardial effusion of a homosexual patent with AIDS.

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PATIENT AND METHODS

Patient

A 45-year-old Japanese homosexual patient with AIDS and suffering from respiratory distress was admitted to the Institute of Medical Science Hospital, University of Tokyo in August 1996. He was diagnosed as an HIV carrier exhibiting *Pneumocystis carinii* pneumonia. His CD4 cell count was 8 cells/μl. Pericardial effusion was noted in December 1996, from which lymphoma cells were identified. CHOP chemotherapy decreased pericardial effusion, but the patient died in May 1997 from a generalized cytomegalovirus infection. No pericardial effusion or lymphoma masses were noted at autopsy.

Establishing the Cell Line

Lymphoma cells in the pericardial effusion were collected and grown in RPMI1640 culture medium supplemented with 20% FBS, 10 ng/ml insulin (Sigma, St. Louis, MO) 10 ng/ml transferrin (Sigma), and 10% supernatant of filtered pericardial effusion. Supplementation of the pericardial effusion was gradually reduced during passages until the cells could grow without it. Single-cell cloning was performed successfully. The cell line thus obtained was named TY-1.

Another PEL cell line, BCBL-1 [Renne et al., 1996], which harbors HHV-8 throughout passaging, was used as a control.

Immunophenotypic Analysis

The immunophenotyping of the TY-1 and original lymphoma cells was performed by flow cytometry, using a FACScan fluorescent activated cell sorter (Becton Dickinson, Mountain View, CA). For the first antibodies of the FACS analysis, we used mouse monoclonal antibodies, including CD3 (Leu4, Becton Dickinson), CD4 (Leu3a, Becton Dickinson), CD5 (Leu-1, Becton Dickinson), CD8 (Leu-2a, Becton Dickinson), CD10 (CALLA, DAKOPATTS, Denmark), CD11b (2LPM19c, DAKOPATTS), CD11c (FK24, Nichirei Corporation, Tokyo), CD14 (Leu-M3, Becton Dickinson), CD20 (L26, DAKOPATTS), CD21 (1F8, DAKOPATTS), CD30 (BerH2, DAKOPATTS), CD34 (BIRMA-K3, DAKOPATTS), CD56 (Leu19, Becton Dickinson), CD68 (KP-1, DAKOPATTS), CD79a (DAKOPATTS), IgG (DAKO-IgG, DAKOPATTS), IgM (DAKO-IgM, DAKOPATTS), IgD (DAKO-IgD, DAKOPATTS), Ig kappa (Kappa Light chain, DAKOPATTS) and Ig lambda (Lambda Light chain, DAKOPATTS). Fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ fragment of goat anti-mouse immunoglobulin (DAKOPATTS) was used as the second-phase reagent.

Southern Blot Analysis

Southern blot hybridization was performed to determine the gene rearrangements of the IgH, T-cell receptor (TCR) and c-*myc* genes. This method was also used to demonstrate the presence of HHV-8 and EBV genomes. The following probes were used: IgH joining region (J_H) probe for IgH [Korsmeyer et al., 1981]; TCR β gene for T-cell receptor [Yanagi et al., 1984]; MC41 5P probe for c-*myc* gene [Dalla-Favera et al., 1983]; $KS330_{233}$ internal probe for the HHV-8 PCR product [Chang et al., 1994]; and a ^{32}P-labeled 60-mer oligonucleotide probe for HHV-8-terminal repeat (TR) (5′-gtg tgt gag cct gtt tgg ggg agc ctc ctc agt gct tgc tac gtg gag ccc tgg aca cta-3′) [Russo et al., 1996] and for a 1.9 kbp *Xho*I fragment of EBV [Raab-Traub and Flynn,1986]. The standard method of Southern was used for this analysis [Southern, 1975].

Cytogenetic Analysis

Chromosome slides were prepared by short-term culture method. In brief, cells were suspended in a petri dish filled with the standard growth medium supplemented with 20% FBS and 0.02 μg/ml colcemid. The cells were incubated for 3–5 hours at 37°C and harvested. Karyotype analysis was carried out at different passages choosing 20 well-banded metaphases each time.

PCR Analysis

We used PCR analysis to determine whether HIV-1, EBV or HHV-8 genomes were present. All the DNA samples were first confirmed to be amplifiable by introducing primers specific for a conserved region of human β-actin gene. The following PCR primers were synthesized and used: a 422 bp V3 region of HIV *env* (5′-ata agc ttc aat gta cac atg gaa tt-3′ and 5′-atg aat tca tta cag tag aaa aat tcc c-3′) [Wolfs et al., 1992], a 129 bp W region of EBV (5′-cca gac agc agc caa ttg tc-3′ and 5′-ggt aga aga ccc cct ctt ac-3′) [Uhara et al., 1990] and a 233 bp $KS330_{233}$ region of HHV-8 (5′-agc cga aag gat tcc acc at-3′ and 5′-tcc gtg ttg tct acg tcc ag-3′) [Chang et al., 1994]. Each PCR reaction was performed using 0.2 μg of genomic DNA, as described previously [Uhara et al., 1990; Wolfs et al., 1992; Chang et al., 1994].

In situ Hybridization

In situ hybridization (ISH) was performed to detect EBV and HHV-8 genomes. EBV-encoded small RNA1 (EBER1) and HHV-8-TR were selected as the targets of ISH. We used a digoxygenin (DIG)-labeled 30-base oligonucleotide, 5′-aga cac cgt cct cac cac ccg gga ctt gta-3′, as a probe for the EBER1 gene. The probe was labeled with a DIG Oligonucleotide 3-Tailing Kit (Boehringer Mannheim, Mannheim, Germany) according to the specification sheet of the labeling kit. Cytospined cells on slides were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.2 for 5 minutes. The procedure of ISH was previously reported [Uhara et al., 1990]. Hybridization was detected with nitro blue tetrazolium salt (NBT).

To detect HHV-8-TR DNA, a DIG-labeled 60-base oli-

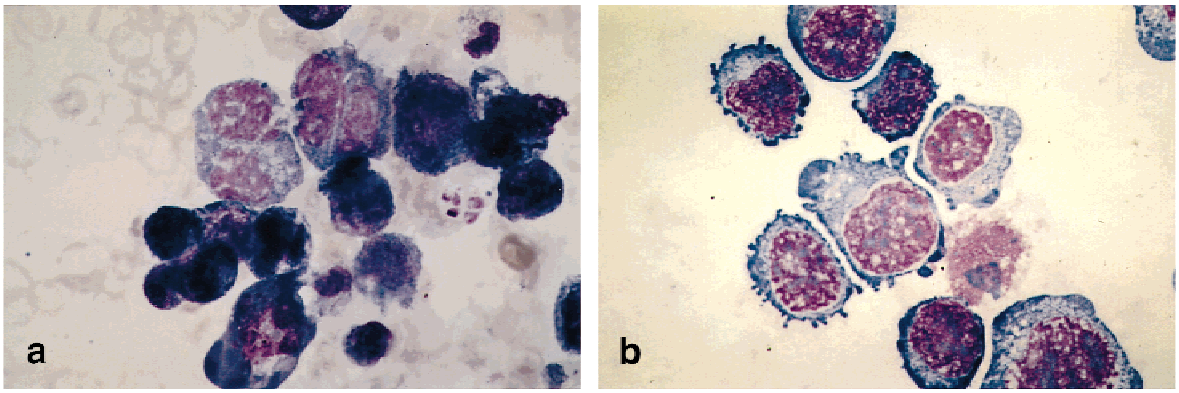

Fig. 1. Morphology of the TY-1 cell line: **(a)** The original pericardial lymphomatous effusion (Gimsa staining); **(b)** Gimsa staining of TY-1.

gonucleotide (described above in the paragraph of Southern blot analysis) was used. In this procedure, the DNA was denatured at 95°C for 5 minutes on a hot plate before hybridization.

TPA or *n*-Butyrate Treatment

To induce HHV-8 virus production, TY-1 and BCBL-1 were cultured for 48 hours in 20 ng/ml TPA or 3 mM *n*-butyrate, as described in a previous report [Miller et al., 1997].

Electron Microscopy

Electron microscopy was performed to demonstrate the presence of viral particles. TPA-treated TY-1 was pelleted by centrifugation, fixed with 4% paraformaldehyde, cut into 1-mm cubes and embedded in epon. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with a JEOL transmission electron microscope (JEOL, 100C, Japan Electric, Tokyo, Japan).

Northern Blot Analysis

We used Northern blot analysis to estimate the amount of T1.1/*nut*-1 mRNA, a putative HHV-8-encoded transcript expressed selectively in the lytic phase [Russo et al., 1996; Bais et al., 1998]. Total RNA was extracted from TY-1 and BCBL-1 using an ISOGEN RNA extraction kit (Nippon Gene, Tokyo); 5-μg RNA samples were separated on a 1.4% formaldehyde-containing agarose gel, transferred to a nylon filter, and hybridized with probe. A 50-mer oligonucleotide probe specific for T1.1/*nut*-1 (5′-gag cgc tcc cag ctg ccg cac acc act tta gtc caa tgt tct tac act ac-3′) was synthesized and labeled with ^{32}P using T4 nucleotide polymerase [Zhong et al., 1996] and used as the antisense probe.

Immunofluorescent Assay for HHV-8 Latency-Associated Nuclear Antigen

Immunofluorescent assay (IFA) was carried out to detect the HHV-8-encoded latency-associated nuclear antigen (LANA), as described previously for the BCP-1 and HBL-6 cell lines [Gao et al., 1996; Kedes et al.,

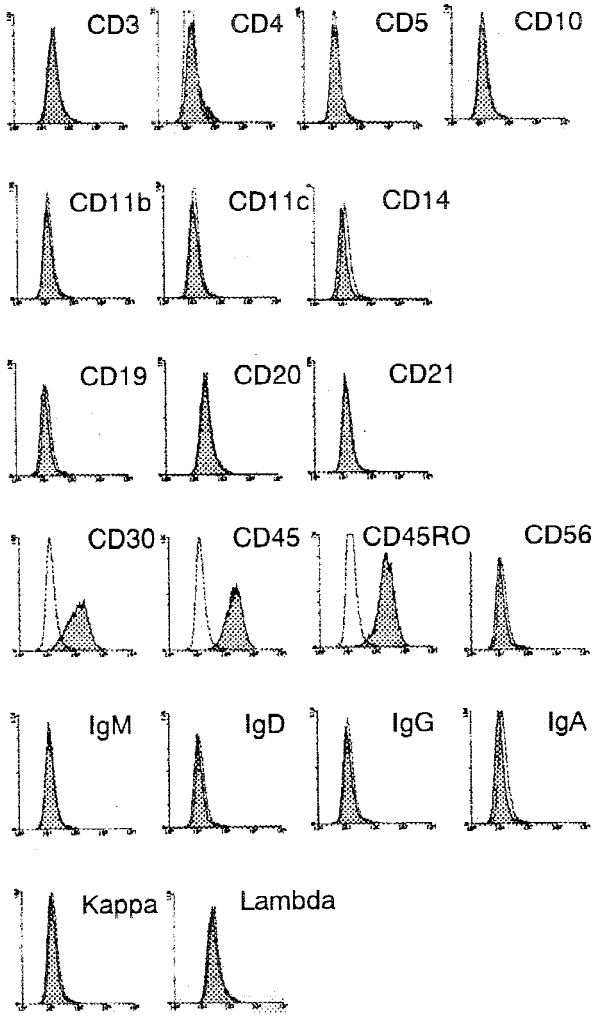

Fig. 2. Flow cytometer profile of the TY-1 cell line. Representative example of fluorescent activated cell sorter profiles of Ty-1. Fluorescence intensity is expressed on a logarithmic scale. Black histograms represent antibody-stained cells; white histograms are isotype-specific controls for each antibody. TY-1 cells lack T- and B-cell and macrophage markers but express CD30, 45 and 45RO cell surface antigens.

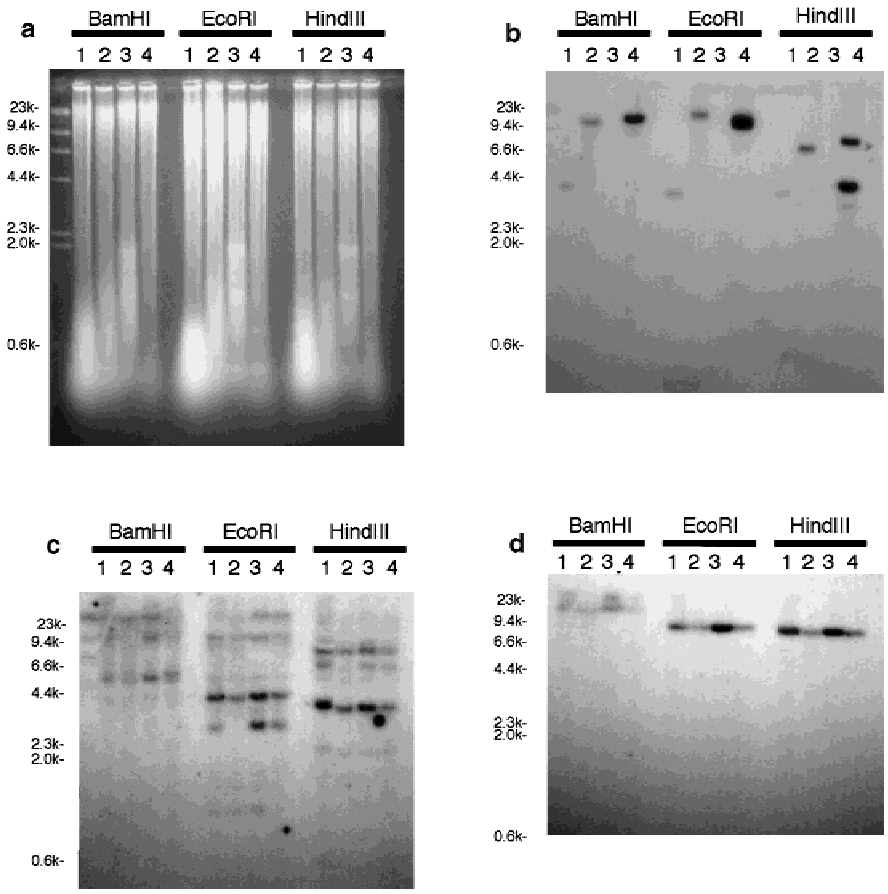

Fig. 3. Gene rearrangement analysis. Southern blot analysis of the TY-1 cell line using an Ig JH probe **(b),** T-cell receptor (TCR) β probe **(c)** and c-myc probe **(d)**. 10μg DNA extracted from each sample was digested with BamHI/ EcoRI/ HindIII, and electrophoresed. **(a)** shows the etidium bromide staining of the electrophoresied DNA. **Lane 1:** BCBL-1 cell line; **lane 2:** the cells in original pericardial effusion; **lane 3:** the TY-1 cell line; **lane 4:** Jurkat cell line (T-cell line). In (b), neither rearrangement band nor germ band is observed in TY-1. TCRβ and *c-myc* rearrangement bands were not detected in either the original pericardial effusion or the TY-1 cell line (c, d).

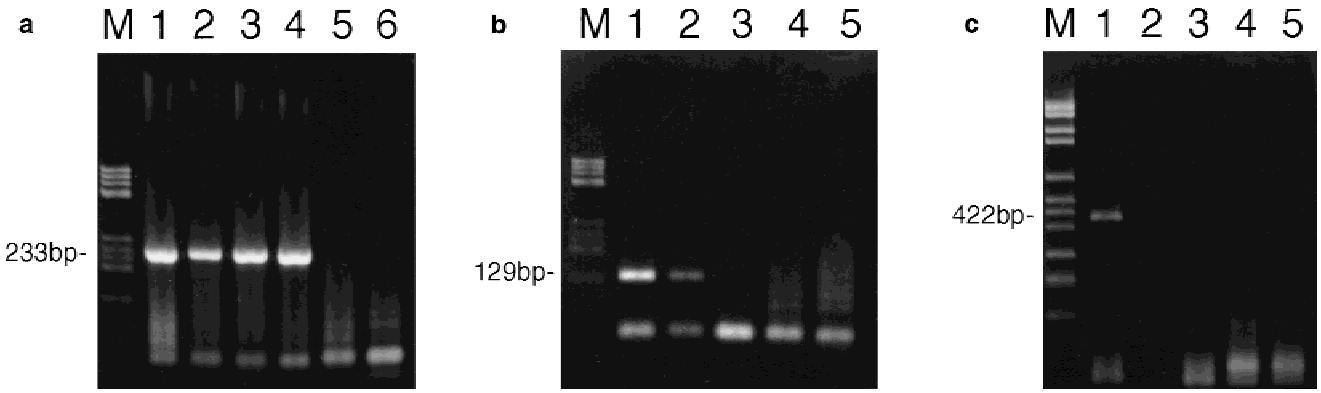

Fig. 4. Viral DNAs in the TY-1 cell line demonstrated by PCR. **(a)** Detection of human herpesvirus 8 (HHV-8). The KS330233 region of HHV-8 was amplified by PCR. **Lane 1:** DNA extracted from BCBL-1; **lane 2:** AIDS-associated Kaposi's sarcoma tissue obtained from another patient: **lane 3:** cells in the original pericardial effusion; **lane 4:** the TY-1 cell line; **lane 5:** Namalwa, Epstein–Barr (EBV)-positive/ HHV-8-negative cell line established from Burkitt's lymphoma (used as a negative control); **lane 6:** water; M: molecular weight marker. HHV-8 was detected in TY-1 and the original primary effusion lymphoma (PEL) cells from which they were cloned. **(b)** EBV genome. The EBV W-region (129 bp) was amplified. **Lane 1:** Namalwa; **lane 2:** cells in the original pericardial effusion; **lane 3:** the TY-1 cell line; **lane 4:** Molt4; **lane 5:** water. EBV was not detected in the TY-1 cell line, while a weak band was observed in the original PEL cells. **(c)** HIV genome. **Lane 1:** AIDS-LN, DNA extracted from the lymph node of a patient with AIDS (positive control): **lane 2:** BCBL-1; **lane 3:** cells in the original pericardial effusion; **lane 4:** the TY-1 cell line; **lane 5:** Namalwa. No sequence of HIV was detected in TY-1 or in cells of the original PEL.

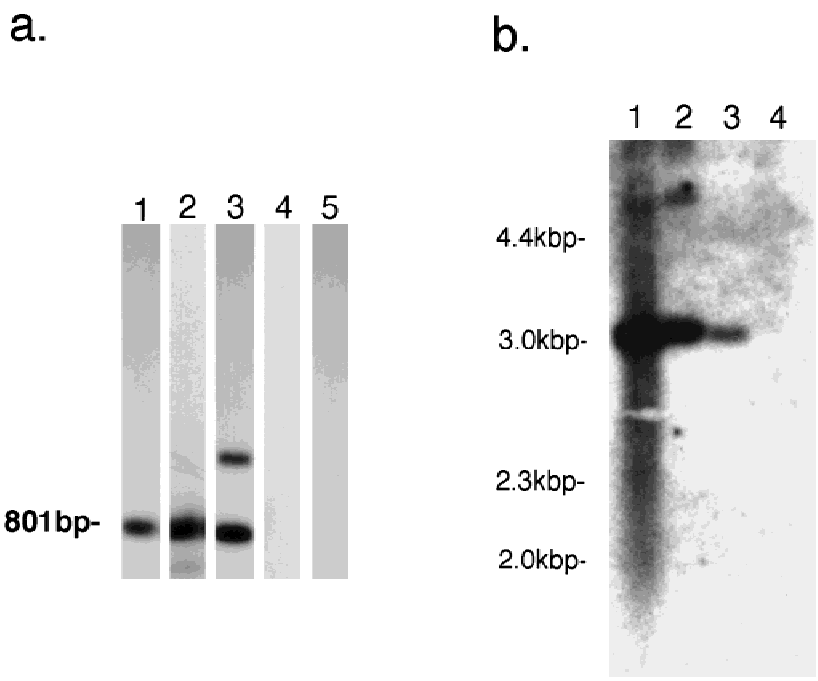

Fig. 5. Human herpesvirus 8 (HHV-8) and Epstein–Barr virus (EBV) genomes demonstrated by Southern blot analysis. **(a)** Using a ^{32}P-labeled 60-mer oligonucleotide probe for HHV-8-TR. **Lane 1:** DNA extracted from BCBL-1; **lane 2:** the TY-1 cell line; **lane 3:** cells in the original pericardial effusion; **lane 4:** Raji; **lane 5:** Jurkat. DNAs were digested with *Not*I. HHV-8 was detected in the TY-1 cell line and the original primary effusion lymphoma cells from which they were cloned. Raji and Jurkat cell lines were used as negative controls. **(b)** DNAs were digested with *Xho*I and hybridized with a ^{32}P-labeled probe for EBV-TR. **Lane 1:** DNA extracted from B95-8; **lane 2:** Namalwa; **lane 3:** cells in the original pericardial effusion; **lane 4:** TY-1 cell line. EBV was detected in B95-8, Namalwa and the original lymphoma but not in TY-1.

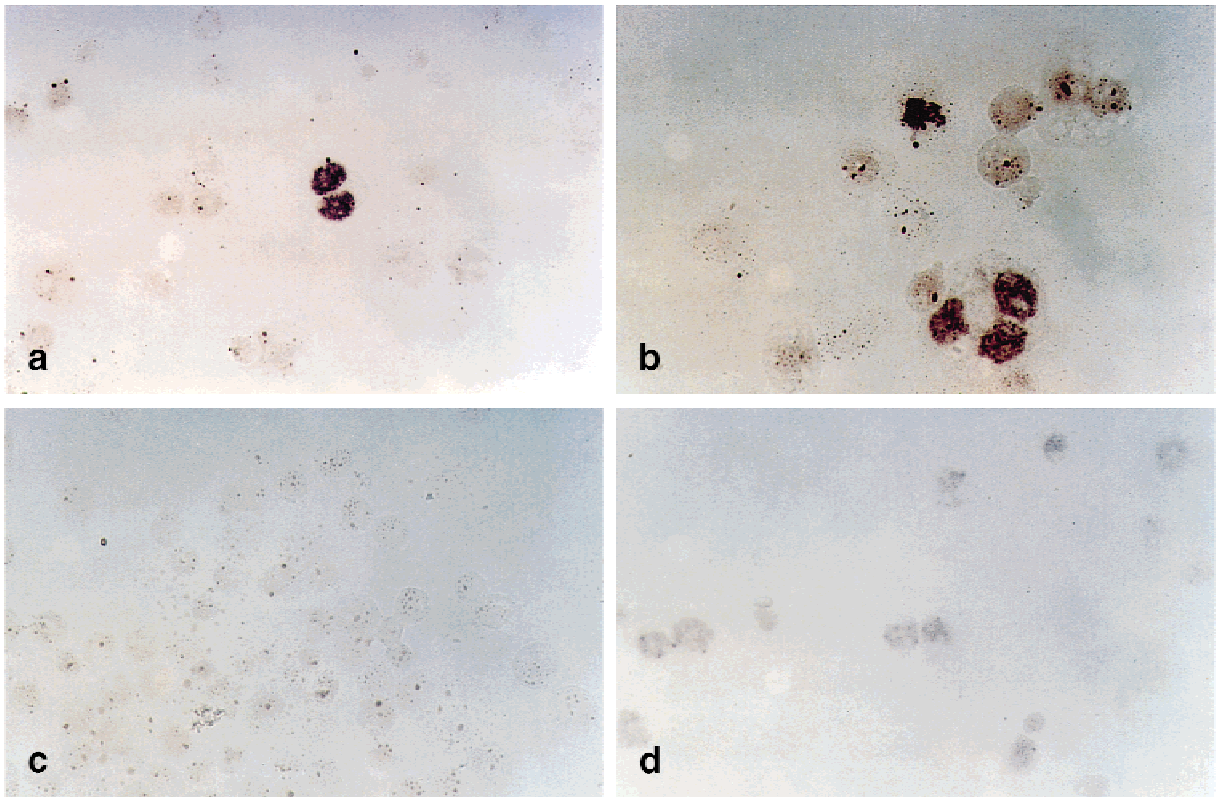

Fig. 6 Localization of human herpesvirus 8 (HHV-8) in the TY-1 cell line. In situ hybridization using a digoxygenin-labeled 60-mer oligonucleotide probe for HHV-8-TR in cell lines: **(a)** BCBL-1; **(b)** TY-1; **(c)** Raji; and **(d)** Ramos (an Epstein–Barr-negative/HHV-8-negative B-cell line established from Burkitt's lymphoma.) Dense signals were observed in the nuclei of some but not all TY-1 and BCBL-1 cells.

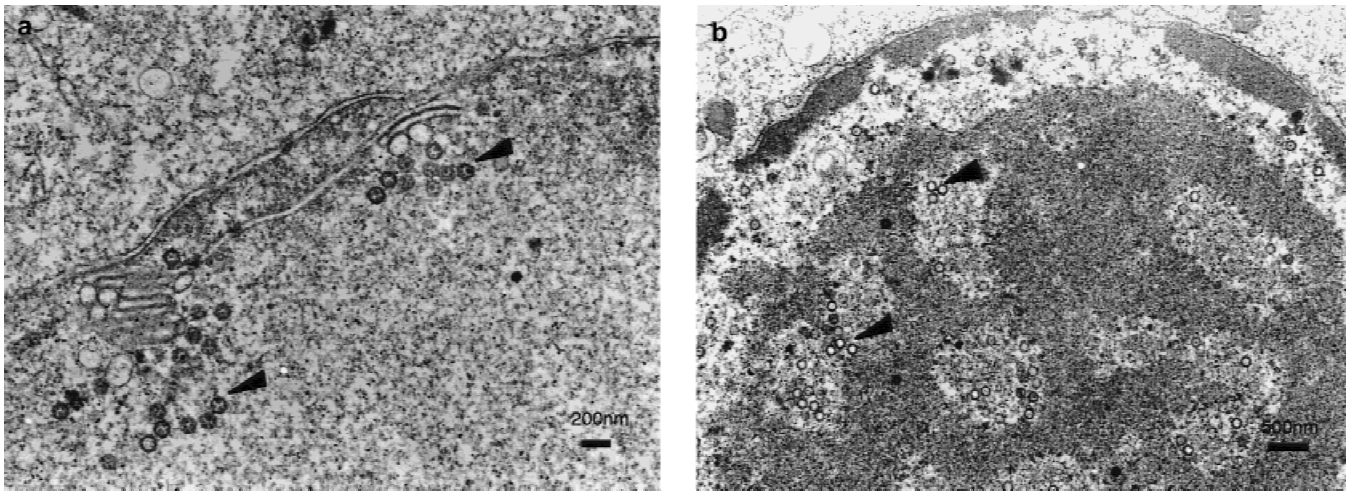

Fig. 7. Electron microscope observation of human herpesvirus 8 in the TY-1 cell line. High magnification views revealed the presence of numerous viral particles with a diameter of 100 nm in the nuclei of the cells.

1996; Moore et al., 1996]. Sera derived from patients with AIDS who were suffering from Kaposi's sarcoma were collected, diluted to 1:200 in PBS-2% fetal calf serum and submitted for IFA. A rabbit anti-human IgG fluorescein isothiocyanate conjugate (DAKOPATTS) was used as the second antibody.

Results

Morphology of TY-1

The pericardial effusion derived from the patient contained a substantial number of large atypical cell with immunoblastic morphology (Fig. 1a). TY-1, the cell line established from this PEL case, showed the typical immunoblastic features characterized by large size (15–25 μm in diameter), one or two large nuclei with heterochromatin and prominent nucleolus and pyroninophilic cytoplasm (Fig. 1b).

Immunophenotype

Flow cytometer analysis revealed that TY-1 was positive for CD30, CD45 and CD45RO, and negative for CD3, CD4, CD5, CD8, CD10, CD11b, CD11c, CD14, CD20, CD21, CD56, CD68, Ig heavy chains γ, μ, δ, α, and light chains κ and λ (Fig. 2). This immunophenotypic feature was identical to that of the original pericardial effusion (data not shown).

Gene Rearrangement Analysis

Southern blot analysis of TY-1 with a JH probe did not demonstrate any bands, including the germ band, while the original specimen from the pericardial effusion exhibited an apparent single germ cell band (Fig. 3a,b). We speculate that the absence of the germ cell band in TY-1 was caused by the deletion of both alleles that react with this probe, suggesting the possibility of clonal B-cell proliferation. Rearrangements of TCR β and c-*myc* were not observed in TY-1 or the original specimen (Fig. 3c,d).

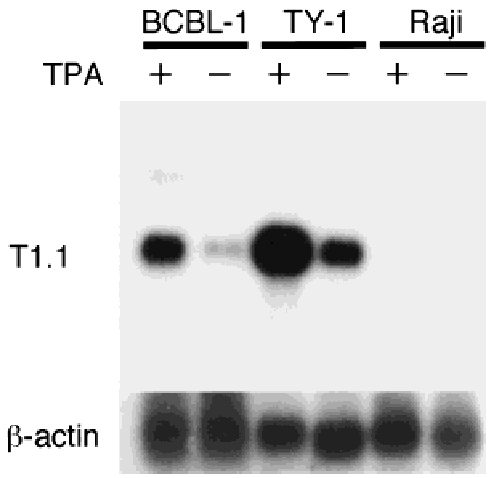

Fig. 8. Expression of T1.1/*nut-1* mRNA in the TY-1 cell line demonstrated by Northern blot analysis. **Upper:** Using a ^{32}P-labeled 60-mer oligonucleotide probe for T1.1; **lower:** Using a ^{32}P-labeled probe for β-actin. Treatment with tetradecanoylphorbol acetate induced the expression of T1.1/*nut-1* mRNA in TY-1 as well as in the BCBL-1 cell line.

Karyotype Analysis

By analysing over 20 well-banded metaphases, the representative karyotype of TY-1 was concluded to be 50,XYq-,+7,+8,+11,+15 (data not shown).

Expression of Viral Genomes

On PCR, the DNA extracted from the original specimen exhibited bands compatible with EBV and HHV-8 DNA sequences. Only HHV-8, and not EBV DNA bands, however, were detected in TY-1 (Fig. 4). Bands compatible with HIV-1 were not detected in TY-1 or the original specimen. On Southern blot analysis, a high load of HHV-8 genome was demonstrated in both TY-1 and the original. An additional band of 1.5 kbp was identified in original PEL cells but the significance of this remains unclear (Fig. 5a). EBV was detected only

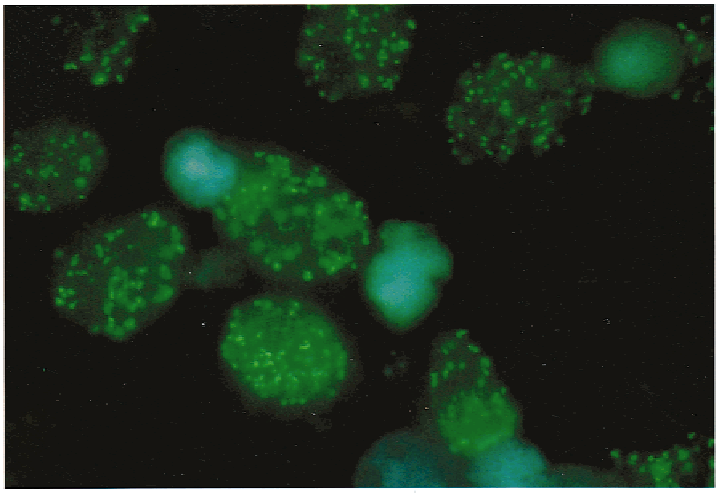

Fig. 9. Demonstration of the human herpesvirus-encoded latency-associated nuclear antigen in the TY-1 cell line. Immunofluorescence assay of TY-1 using the serum from a patient with AIDS-associated Kaposi's sarcoma as first antibody. Multiple fluorescent spots were observed in both TY-1 and BCBL-1 cell lines.

in the original specimen and not in TY-1 (Fig. 5b). The viral load of EBV in original pericardial cells, as shown by Southern blot analysis, was less than in the Namalwa cell line, which is known to carry one or two copies of the EBV genome in each cell (Fig. 5b).

In Situ Localization of HHV-8

Using ISH, HHV-8 was detected in the nuclei of only around 1% TY-1 cells (Fig. 6), but this proportion apparently increased to around 10% with TPA treatment (data not shown). Electron microscope analysis revealed numerous viral particles of about 100 nm in diameter in the nuclei of these cells (Fig. 7).

Demonstration of HHV-8-Encoded Gene Products

T1.1/*nut*-1 is an mRNA reported to be expressed in the lytic phase of HHV-8 infected cells [Zhong and Ganem, 1997]. Northern blot analysis demonstrated that, after TPA treatment, TY-1 expressed T1.1/*nut*-1 mRNA at a level about 5 times greater than TPA-treated BCBL-1, as measured by BAS-2000 densitometry (Fujifilm, Tokyo, Japan) (Fig. 8). On IFA, sera from AIDS patients carrying Kaposi's sarcoma reacted with nuclear antigen in TY-1 in the speckled pattern (Fig. 9). This staining pattern matched to that of LANA [Moore et al., 1996]. Comparing the result of ISH for HHV-8-TR, much more cells were positive for LANA. It is because ISH for HHV-8-TR detects cells in lytic phase exclusively.

DISCUSSION

In this article we described a new HHV-8-positive and EBV-negative PEL cell line, TY-1. While several HHV-8-positive PEL cell lines have been reported, a large portion of them are known to be coinfected with EBV. Only three HHV-8-positive/EBV-negative PEL cell lines have been reported so far [Cesarman et al., 1995b; Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996].

In our study, the EBV genome was first identified in original pericardial cells by Southern blot analysis. The amount of EBV genome, however, remained small, far less than that of the control EBV-infected cell line, which is known to carry 1 or 2 copies per cell. This suggests that only a portion and not all of the original PEL cells were infected with EBV. By contrast, the amount of HHV-8 in original PEL cells was high, on a level equal to the cloned cell line, TY-1, and the control HHV-8-positive cell line, BCBL-1.

Considering that all the TY-1 cells were positive for the HHV-8-encoded protein, LANA, it is likely that the original PEL cells and TY-1 carry roughly the same amount of HHV-8. Thus, it might be speculated that there were both HHV-8-positive/EBV-negative and HHV-8-positive/EBV-positive clones in the original PEL, while only the former was cloned as the TY-1 line.

While the immunophenotypic analysis could not specify the lineage of TY-1, Southern blot analysis using a JH probe strongly suggested its B-cell origin. the germ band, which should be present in all lymphoid or nonlymphoid cells, was shown by Southern blot analysis to be lost in TY-1. This unique finding can be explained only by the loss of both alleles at this site as a result of clonal rearrangement of the IgH gene in such a way that JH is lost at this site. This loss can also be seen in less than 5% of B-cell neoplasms (personal observations).

Other molecular and immunophenotypic characteristics of this cell line, such as the absence of c-*myc* gene rearrangement, loss of B-cell and T-cell markers and the expression of CD45 (leukocyte common antigen), correspond to those of previously reported PEL cell lines. Furthermore, we noted in the present study that TY-1 expressed CD30 (Ki-1 antigen, one of the TNF-receptor type receptor protein) which is expressed in T

or B cells in activated state, Reed-Steinberg cells in Hodgkin's disease and anaplastic large cell lymphoma (ALCL) cells. This molecule was expressed in the original PEL, and its expression was not decreased in TY-1. Some PELs were reported to be positive for CD30 [Nador et al., 1996], so it may be one of the immunophenotypic characteristics of the PEL.

The increased quantity of T1.1/*nut*-1, a lytic-phase gene product expressed after the TPA and butyrate treatments, was also seen in BCBL-1, the reported HHV-8-positive/EBV-negative cell line. Cytogenetic analysis exhibited other clonal abnormalities to which we cannot ascribe any specific significance at present. The cellular characteristics of TY-1 thus accord well with other PEL cell lines. This may not necessarily lower the value of this line, however, because the characteristics described in this paper are superficial and do not reveal anything about the tumorigenetic mechanism of TY-1. Our knowledge of PEL is still limited and the addition of this new cell line will contribute at least in two aspects, that is, the study of cellular events in PEL, and the study of viral protein expression in PEL.

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